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A NOVEL, RAPID ASSAY FOR DETECTION AND DIFFERENTIATION OF SEROTYPE-SPECIFIC ANTIBODIES TO VENEZUELAN EQUINE ENCEPHALITIS COMPLEX ALPHAVIRUSES

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Abstract. An epitope-blocking enzyme-linked immunosorbent assay was developed for the rapid differentiation of serologic responses to enzootic variety IE and ID versus epizootic variety IAB and IC strains of Venezuelan equine encephalitis (VEE) virus. Two monoclonal antibodies that differentially recognize epizootic versus enzootic VEE virus epitopes were used to measure the serotype-specific blocking abilities of antibodies in sera of naturally infected humans, equines, and bovines, as well as in experimentally infected equines. The assay is simple, species-independent, rapid, and sensitive, and will improve surveillance for VEE emergence. It could also be used to determine the epidemic potential of a VEE virus following an intentional introduction for bioterrorism.

INTRODUCTION

Venezuelan equine encephalitis viruses (VEEVs) include re-emerging epizootic and epidemic strains that belong to the genus *Alphavirus* in the family Togaviridae.[1] A group of closely related viruses including Everglades, Mucambo, Tonate, Cabassou, and Rio Negro, which were originally classified as subtypes in the VEE serocomplex,[2] are now considered distinct species.[1] Venezuelan equine encephalitis virus and related VEE complex viruses are naturally transmitted among vertebrate hosts by mosquitoes, but many are also highly infectious via the aerosol route and have caused many laboratory infections.[3] Epizootic strains have caused numerous outbreaks of human and equine disease in the Americas since the 1920s, and recent epidemics indicate that VEEV continues to pose a serious threat to public health.[4] Recently, attention and research has focused on the potential use of VEEV as a biologic weapon.[5] Compounding the fears of health care providers, no effective treatment exists for infected equines or humans, although the attenuated vaccine virus strain TC-83, derived from an epizootic variety IAB strain, is used for equines in disease-endemic locations or during epizootics, and for at-risk humans.[4]

The VEE complex viruses have plus sense RNA genomes of approximately 11.4 kb in length. The 5′ two-thirds of the genome encode four nonstructural proteins (nsP1–nsP4) that are involved in viral RNA replication.[6] The three structural proteins (capsid and E2 and E1 envelope glycoproteins) are transcribed from a 26S subgenomic RNA that is identical to the 3′ one-third of the genome. The major antigenic determinants that define VEEV subtypes and varieties are located on the E2 envelope glycoprotein, and include neutralization and several different, subtype- and variety-specific epitopes.[7]

The VEE complex alphaviruses are grouped into six antigenic subtypes based primarily on E2 protein epitopes. Subtypes II-VI and varieties ID-IF, many of which are now considered species distinct from VEEV, are enzootic viruses that are generally not associated with major epidemics or equine epizootics, but can cause fatal human disease.[4] Subtype I, varieties AB and C VEEV cause severe disease in both humans and equines, and are generally isolated only during epidemics and epizootics.[8] Infection of humans usually produces a "flu-like" disease. The less common encephalitic form of human VEE is characterized by disorientation, ataxia, mental depression, and convulsions, and can be detected in up to 14% of infected individuals, especially children.[9] Overall mortality rates during outbreaks rarely exceed 1%, but neurologic sequelae following human VEE are common.[10] Long-term immunosuppression in patients who recover from VEEV infection has also been reported.[11]

Diagnosis of VEE relies on virus isolation from acute phase serum or from spinal fluid of human or animal origin, or on detection of VEEV-specific IgM in the cerebrospinal fluid in cases of encephalitis.[12] Four-fold or greater increases in VEEV-specific antibodies can also be used to confirm an infection retrospectively. An IgM capture enzyme-linked immunosorbent assay (ELISA), as well as a monoclonal antibody (MAb)–based antigen-capture ELISA have been developed for detecting antibodies to alphavirus[13–15] and are used for diagnosis of infections with encephalitic alphaviruses domestic to the United States such as EEEV and WEEV (http://www.cdc.gov/ncidod/dvbid/arbor/arbdet.htm).

Identification of the subtype and variety of antibodies to VEEV in equines, humans, or rodent reservoir hosts can be critical for determining the potential of a naturally circulating or intentionally introduced strain to cause an epidemic via equine amplification. Although IgM assays readily differentiate infections with different alphaviruses, discrimination of subtype- or variety-specific antibodies to VEEV is difficult. The best methods currently available for discriminating enzootic- and epizootic-specific antibodies are plaque reduction neutralization tests (PRNTs). However, due to the similarity of the neutralization domains of closely related variety ID/IE and IAB/C VEEV strains, the degree of cross-neutralization is very high making the final diagnosis using the PRNT difficult. To overcome these problems, we developed an epitope-blocking-ELISA, similar to previously published assays for detection of antibodies to flaviviruses[16–18] and alphavirus,[19] which is able to distinguish between infections with enzootic, variety ID/E/F and epizootic, variety IAB/C VEEV strains.

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MATERIALS AND METHODS

Viruses and cell cultures. Four representative VEEV strains were used: variety IAB vaccine virus strain TC-83,[20] enzootic variety ID strain 66637 from Venezuela,[21] epizootic variety IC strain 243937 from Venezuela,[22] and enzootic variety IE strain 68U201 from Guatemala.[23] All virus stocks were prepared in baby hamster kidney (BHK) cells or Vero cells obtained from the American Type Culture Collection (Manassas, VA) using Eagle's minimal essential medium supplemented with 5% fetal bovine serum.

Antigen preparation. Virus stocks purified from BHK cells using polyethylene glycol/NaCl precipitation and rate/zonal ultracentrifugation on sucrose density gradients.[24] They were stored at -80°C in EDTA-free, 50 mM bicarbonate buffer (pH 9.6) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and used as antigens.

Monoclonal antibodies. The MAb 1A3A-5, which reacts only with epizootic variety IAB or IC VEEV, and MAb 1A1B-9, which reacts only with enzootic variety ID and IE in immunofluorescence assays, were described previously[25] (Table 1). All MAb stocks were diluted in water to a concentration of 1 mg/mL.

Monoclonal antibody–based epitope blocking ELISA. Optimal concentrations of viral antigens were estimated by titration with each MAb; 100 μL each of two-fold serial dilutions (beginning at 1:100) of virus in bicarbonate buffer were added to each well of a Nunc Immuno PolySorp 96-well plate (Nalge Nunc International, Rochester, NY) and incubated overnight at 4°C. The plate was then washed with buffer (phosphate-buffered saline with 0.1% Tween 20 [Sigma, St. Louis, MO]) and blocked with blocking buffer (phosphate-buffered saline, 0.1% Tween 20, 1% bovine serum albumin). Fifty microliters of two-fold serial dilutions of each MAb (beginning at 1:100) were added and the plate was incubated for one hour at 37°C. After four additional washes, 50 μL of two-fold serial dilutions (beginning at 1:1,000) of horseradish peroxidase–conjugated rabbit anti-mouse IgG (Sigma) in blocking buffer was added to each well and incubated for one hour at 37°C, followed by four washes. Two hundred microliters of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added to each well and incubated for 30 minutes at 37°C. The reactions were stopped by adding 0.5 M H_2SO_4 and plates were read at 450 nm in an ELISA plate reader. The highest optical density (OD) values from each titration were determined as the optimal antigen and antibody dilutions.

Epitope-blocking ELISAs were performed as described previously[18] with minor modifications. Briefly, purified virus was diluted in buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6). Each well of a 96-well plate surface was coated with 100 μL of diluted antigen. Coating buffers were used as controls for calculation of background OD. Coated plates were incubated overnight at 4°C and washed four times with 200 μL of wash buffer. Two hundred microliters of blocking buffer was then added to each well and incubated for one hour at 37°C to saturate non-specific binding sites. After four washes, 50 μL of diluted serum (1:4, 1:12, and 1:36) were added to each well and incubated for one hour at 37°C and then washed four times with wash buffer. The variety-specific murine MAbs were diluted in blocking buffer, added to the antigen, and incubated for one hour at 37°C in a final volume of 50 μL. Plates were washed again four times, then 50 μL of horseradish peroxidase–conjugated rabbit anti-mouse IgG (Sigma) at a 1:5,000 dilution in blocking buffer were added to each well and incubated for one hour at 37°C,

TABLE 1

Serologic reactions of human sera from Mexico and Peru using a blocking enzyme-linked immunosorbent assay and plaque reduction neutralization tests*

Serum number	Country, year, VEE variety	% Inhibition† of MAb 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of MAb 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IE/ID¶	Variety IAB/C
503	Mexico, 2001	10.3	16.0	–6.6	**94.3**	**81.7**	**68.6**	**320**	**40**
525	Mexico, 2001	10.5	5.2	–8.5	**82.0**	**64.6**	**47.0**	**320**	**160**
528	Mexico, 2001	12.3	9.6	–10.7	**62.1**	**28.5**	18.1	**320**	**160**
545	Mexico, 2001	4.3	–1.1	–8.9	**74.7**	**42.7**	17.4	**160**	**40**
168	Mexico, 2001	8.7	4.5	–9.6	**84.3**	**62.2**	**48.3**	**160**	**80**
505	Mexico, 2001	–13.4	–12.7	–27.2	–6.1	–18.7	–11.8	< 20	< 20
470	Mexico, 2001	–9.1	–8.2	–12.2	0.8	8.9	22.0	< 20	< 20
486	Mexico, 2001	–6.2	–8.6	–10.7	18.7	9.8	14.9	< 20	< 20
504	Mexico, 2001	–9.3	14.4	–13.3	6.9	–16.2	–9.4	< 20	< 20
FSL0206	Peru, 2000, ID#	18.8	9.5	–8.0	**65.3**	**51.0**	**29.0**	**320**	**80**
IQT8231	Peru, 1998, ID#	7.8	4.1	–13.3	**33.5**	**27.6**	**29.5**	**40**	**20**
IQT7660	Peru, 1998, ID#	**38.2**	–4.3	–9.5	**62.9**	**44.3**	**52.0**	**20**	**20**
IQD4155	Peru, 1998, ID#	–1.1	4.5	–4.3	**44.3**	**47.8**	**47.9**	**40**	< 20
IQD2652	Peru, 2002, ID#	5.5	11.2	1.0	**73.5**	**56.3**	**51.9**	**320**	**40**
IQU1617	Peru, 1999, ID#	**34.8**	14.3	–3.9	**85.2**	**77.2**	**51.5**	**40**	**20**
IQT7172	Peru, 1998, ID#	18.8	14.1	4.2	11.1	**69.0**	**55.9**	**80**	**20**
FSL0241	Peru, 2000, ID#	16.4	1.4	1.7	**50.1**	16.7	**18.9**	**320**	**20**
UT445	Colombia, 2003, ID#	**55.6**	15.4	13.1	**94.0**	**78.1**	**62.4**	**320**	**80**
FSL0191	Peru, 2000, IIID#	–5.8	–2.5	–24.6	18.4	14.0	13.3	< 20	< 20

* VEE = Venezuelan equine encephalitis.
† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.
‡ Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.
§ MAb 1A1B-9 binding to variety IE strain 68U201 antigen for Mexican samples and to ID-66637 for Peruvian samples.
¶ Peruvian and Colombian samples were tested against the variety ID strain and Mexican samples were tested against the variety IE strain.
# Sera from patients from which virus was isolated and identified.

followed by four washes. Two hundred microliters of 3,3′,5,5′-tetramethylbenzidine were then added to each well and incubated for 30 minutes at 37°C. Reactions were stopped by adding 100 μL of 0.5 M H_2SO_4 and plates were read at 450 nm in an ELISA plate reader. The percent inhibition of the colorimetric reaction caused by sample antibodies blocking binding of the MAb to the antigen was calculated for each serum at each dilution by using the formula % inhibition = 100 – [(TS – B)/CS – B)] × 100, where TS = OD of the test serum, CS = OD of control serum, and B = background OD. The samples were analyzed in duplicate or triplicate using each antigen. Due to the small volumes available, some sera were only tested once with one or two antigens.

Serum samples. To determine the specificity of the epitope-blocking ELISA using variety-specific MAbs, we used well-characterized sera including samples from experimentally infected horses[26] and convalescent human serum samples from well characterized cases with viral etiologies determined by virus isolation and characterization. Additionally, human, bovine, and horse sera containing VEEV-specific neutralizing antibodies were obtained in regions of Mexico (Tabasco and Veracruz States) where only the IE variety circulates and where vaccination is prohibited. Informed consent was obtained from adult human participants and from the parents or legal guardians of minors. The University of Texas Medical Branch and the Naval Medical Research Center Detachment institutional review boards reviewed and approved the project.

Variety IAB/C- and IE-specific equine sera were also obtained from experimental horse infections previously performed.[26,27] Sera were collected between 7 and 15 days postinfection and kept at -80°C until further processing. All sera were heated to 56°C for 30 minutes prior to serologic tests to inactivate complement.

Plaque reduction neutralization tests. The PRNT was performed to detect the ability of serum samples to neutralize VEEV strains as described previously.[28]

RESULTS

Optimization of the blocking ELISA. The optimal concentrations of antigens and MAbs were determined based on maximizing colorimetric reactions and were as follows: IAB vaccine strain TC83 diluted 1:1,000 with IAB/C-specific MAb 1A3A-5 diluted 1:300; ID strain 66637 diluted 1:200 with MAb 1A1B-9 diluted 1:250; and IE virus strain 68U201 diluted 1:3,200 with MAb 1A1B-9 diluted 1:700. Based on previous ELISA blocking assays and negative control values of sera with known experimental infection histories, inhibition values ≥ 25% for the 1A1B-9 MAb and ≥ 30% for the 1A3A-5 MAb, at a 1:12 serum dilution, were chosen as cutoff values for the detection of variety-specific antibodies.

Serologic tests: human samples. Serum samples were obtained from convalescent Peruvian patients[29,30] with a known history of VEEV infection (virus isolation) and from persons living in areas of Mexico with known variety IE VEEV activity.[31]

All human samples that tested positive in an 80% PRNT at a dilution ≥ 1:20 were also positive in the blocking ELISA at a dilution ≥ 1:12 (Table 1). Samples with PRNT titers below the limit of detection (< 1:20) did not have detectable blocking activity in the ELISA. One convalescent serum (FSL0191) tested negative in our blocking assay; however, this sample was from a patient infected with a variety IIID strain in the VEE complex,[29] which is considered a different species of alphavirus (a variant of Mucambo virus).[1] This result indicates that our assay is specific for antibodies to VEE subtype I, and that the antigens we used in our assays (varieties IAB/C and ID) are probably not recognized by antibodies induced by variety IIID infection.

Some samples had cross-reactive inhibitory activity at a 1:4 dilution (IQT 7660, IQU 1617, UT445) when tested against the 1A3A-5 MAb (variety IAB/C-specific) in the blocking ELISA. However, at the next higher dilution (1:12), they were positive in the assay only against the 1A1B-9 (variety IE/D-specific) MAb, confirming the variety ID VEEV infection determined previously by virus isolation and genetic characterization.[29] All Mexican samples that tested positive for VEEV neutralizing antibodies were, in contrast to the PRNT, easily identified as variety IE/ID specific in the blocking ELISA. One sample (FSL0241) was positive at a 1:4 dilution, but negative at the 1:12 dilution in the variety ID-specific test. Because no cross-reactivity was found in the IAB/C-specific test and a variety ID virus was isolated from this patient, we considered it as weakly positive for variety ID. Overall, our serologic results correlated strongly with the clinical/microbiologic data obtained from patients and with the epidemiologic knowledge of VEEV circulation. The detection of enzootic VEEV-specific human antibodies using the blocking ELISA suggested a slightly lower sensitivity but higher specificity when compared with the PRNT.

Equine samples. Serum samples were obtained from experimentally infected horses and from horses living in regions with known VEEV activity and vaccination policies and programs. All horses experimentally infected with variety IE VEEV had neutralizing antibodies exhibiting a high level of cross-reactivity, making it impossible to identify the variety of VEEV responsible for the infection. When the same samples were tested in the blocking ELISA, all but one (DP4) tested positive at the 1:12 dilution in the variety IE/ID assay while testing negative for variety IAB/C (Table 2).

Less uniform results were obtained with the samples from naturally exposed Mexican horses with no known history of vaccination. Three PRNT-positive horses (VER-15, VER-25, and VER-26) exhibited positive blocking activity in the IAB/C assay and all PRNT-positive horses also showed positive results in the IE/D assay. We could not rule out the possibility that some of the IAB/C-positive horses might have been moved from regions with strain TC-83 virus vaccination programs. All PRNT-negative samples were also negative in both blocking assays, indicating a concordance between the two assays (Table 3).

Serum samples from horses experimentally infected with variety IC VEEV, which had no detectable alphavirus-reactive antibodies prior to inoculation and which survived long enough to develop neutralizing antibodies, all tested positive in the IAB/C blocking assay. The same samples did not show blocking activity in the IE/D assay, indicating a high specificity of the assay and its ability to detect epizootic-specific seroconversion early after infection.

Bovine samples. Unlike equines, cattle are not vaccinated against VEE because they do not develop overt disease de-

TABLE 2

Blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test results from horses infected experimentally with Venezuelan equine encephalitis virus (VEEV)

Serum number	Days after VEEV infection (strain variety)	% Inhibition† of MAb 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of MAb 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer*		
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IC	Variety ID	Variety IE
DP1	14 days (IE)	23.7	7.6	13.3	**40.4**	**27.6**	10.0	**320**	**640**	**640**
DP2	14 days (IE)	**38.5**	17.2	12.2	**47.0**	**37.6**	**24.9**	**640**	**640**	**640**
DP3	14 days (IE)	14.0	7.5	3.1	**57.2**	**51.1**	**41.7**	**320**	**640**	**640**
DP4	14 days (IE)	18.3	13.2	11.3	**46.2**	**23.8**	19.0	**160**	**320**	**640**
DP29	14 days (IE)	17.5	16.6	4.0	**42.3**	**32.0**	19.5	**80**	**160**	**640**
DP30	14 days (IE)	**34.5**	13.3	11.2	**48.9**	**36.1**	**27.9**	**160**	**320**	**640**
DP31	14 days (IE)	19.7	5.4	4.4	**40.6**	**34.5**	15.6	**80**	**320**	**640**
836	10 days (IC)	**81.0**	**74.1**	**75.6**	14.1	–3.4	–11.0	**640**	< 20	NT
744	8 days (IC)	**86.3**	**82.3**	**79.4**	–12.5	21.3	3.5	**640**	< 20	NT
968	15 days (IC)	**81.0**	**78.7**	**75.9**	**40.3**	24.9	–8.3	**80**	**640**	NT
876	15 days (IC)	**80.1**	**73.1**	**65.8**	–7.1	–5.1	–5.1	**80**	**80**	NT

* NT = not tested.
† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.
‡ Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.
§ MAb 1A1B-9 binding to variety IE strain 68U201 antigen.

spite being naturally infected.[32,33] They therefore represent excellent sentinels for VEEV surveillance. Bovine sera were collected from Veracruz State, Mexico, where variety IE VEEV-positive equine samples were previously obtained. Based on the results with equine samples (three variety IAB/C-positive horses), we wanted to investigate possible VEEV transmission to other animal species living close to horses. Five of 10 bovine sera had detectable VEEV neutralizing antibodies (Table 4). All but one (VB-14) of these five PRNT-positive samples tested positive in the variety IE/D/F blocking assay, while all 10 samples were negative in the IAB/C assay. These results indicated previous enzootic VEEV infections, probably caused by variety IE, and a slightly lower sensitivity of the blocking ELISA compared with the PRNT.

DISCUSSION

We developed an epitope-blocking ELISA that will be useful for distinguishing the humoral immune responses to enzootic versus epizootic VEEV infections in a variety of animals and in humans. In all cases in which we had prior information about the source of VEEV infection, we were able to make a specific serologic diagnosis. Some of the field serum samples obtained from horses, which are vaccinated with the IAB-derived TC-83 strain in some regions of Mexico, reacted positively in the epizootic variety IAB/C blocking test. However, the majority of the samples were positive only in the enzootic IE/D-specific test, indicating natural exposure. All seropositive bovines from the same region, which are never vaccinated, showed a positive reaction only in the enzootic IE/D assay. These discordant results suggest that 1) equines in some non-endemic regions of Mexico where vaccination is not permitted are vaccinated illegally with strain TC-83; 2) some of the IAB/C-positive horses were moved from the Pacific coast where vaccination is encouraged, or 3) equines develop higher levels of cross-reactive antibodies after field (possibly multiple) exposure to variety IE strains. Horses experimentally infected with variety IE VEEV from Mexico

TABLE 3

Serologic reactions of horse sera collected in 2000 from Veracruz State, Mexico using a blocking enzyme-linked immunosorbent assay and plaque reduction neutralization tests

Serum number	% Inhibition† of MAb 1A3A-5‡ binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition† of MAb 1A1B-9§ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
	1:4	1:12	1:36	1:4	1:12	1:36	Variety IE/ID	Variety IAB/C
VER-6	**43.1**	15.3	11.1	**86.2**	**71.8**	**63.7**	**640**	**40**
VER-12	**46.0**	28.4	12.1	**92.5**	**74.9**	**63.2**	**320**	< 20
VER-20	NT	NT	NT	**83.6**	**87.5**	**63.2**	**320**	**40**
VER-05	24.4	26.5	7.7	**51.9**	**35.2**	**22.2**	**320**	< 20
VER-15	**50.0**	**34.9**	10.0	**63.6**	**34.8**	**36.3**	**160**	**40**
VER-16	**37.2**	13.9	–3.7	**79.2**	**59.4**	21.8	**160**	**160**
VER-25	**45.8**	**31.8**	8.9	**77.4**	**45.0**	**30.9**	**320**	**80**
VER-26	**58.3**	**37.2**	12.9	**71.9**	**65.0**	**37.6**	**320**	**20**
VER-21	–4.0	3.9	–13.4	–2.3	–12.4	–11.5	< 20	< 20
VER-22	–7.0	5.7	–0.6	3.8	–18.9	–16.1	< 20	< 20
VER-24	6.2	–8.3	–7.1	19.8	8.7	1.8	< 20	< 20
VER-34	0.2	–3.0	–11.4	–6.1	–3.9	12.6	< 20	< 20
VER-41	–25.1	–25.1	–47.0	–3.4	–5.5	13.1	< 20	< 20
VER-36	13.2	–6.9	0.0	0.0	1.3	0.0	< 20	< 20

† % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.
‡ Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.
§ MAb 1A1B-9 binding to variety IE strain 68U201 antigen.

TABLE 4
Blocking enzyme-linked immunosorbent assay and plaque reduction neutralization test results from Mexican bovine sera collected in 2003

Serum number	State	% Inhibition* of MAb 1A3A-5† binding (variety IAB/C-specific) at indicated serum dilution			% Inhibition* of MAb 1A1B-9‡ binding (variety IE/ID-specific) at indicated serum dilution			80% Plaque reduction neutralization titer	
		1:4	1:12	1:36	1:4	1:12	1:36	Variety IE	Variety IAB/C
VB-1	Veracruz	–7.4	–16.7	–13.3	24.3	**26.6**	5.2	**160**	20
VB-3	Veracruz	6.8	–22.1	–27.8	**60.6**	**50.2**	14.6	**320**	**160**
VB-7	Veracruz	2.5	–26.3	–34.4	**51.9**	**33.2**	6.7	**160**	**40**
VB-13	Veracruz	**36.1**	13.8	9.8	**59.2**	**51.1**	4.6	**320**	**320**
VB-14	Veracruz	–3.4	–1.8	–1.7	19.1	14.5	–10.6	**160**	**40**
TB-1	Tabasco	9.4	14.1	12.0	8.0	14.5	15.6	< 20	< 20
TB-2	Tabasco	5.8	11.4	11.3	17.5	23.2	11.0	< 20	< 20
TB-3	Tabasco	–1.9	0.0	0.3	10.0	20.5	6.9	< 20	< 20
TB-5	Tabasco	4.1	6.6	5.4	15.2	23.1	–5.7	< 20	< 20
TB-6	Tabasco	–12.4	–11.1	–13.6	–4.3	3.9	–6.3	< 20	< 20

* % inhibition values are means of duplicate or triplicate wells for each sample and positive results are indicated by **bold** numbers.
† Monoclonal antibody (MAb) 1A3A-5 binding to variety IAB/C strain TC-83 antigen.
‡ MAb 1A1B-9 binding to variety IE strain 68U201 antigen.

tested positive only in the IE/D assay, indicating that primary infection produces more enzootic IE-specific antibodies. Additionally, horses experimentally infected with variety IC VEEV developed antibodies detected only in the IAB/C assay, exhibiting no cross-reactivity in the enzootic IE/D assay.

In addition to being considerably faster than the PRNT, a major advantage of the blocking ELISA is the species-independent nature of the test. Unlike some other ELISA formats, antibodies from any species can be tested using the same reagents. Because VEEV infects a wide variety of animals during outbreaks, this advantage can be exploited to use a number of different animals as sentinels. Bovines offer several advantages as described above, but other domestic animals such as dogs and pigs also become infected and seroconvert,[34] and could be used as surrogates for human exposure.

In general, our blocking ELISA exhibited slightly lower sensitivity than the PRNT. This lower sensitivity may reflect that the blocking ELISA relies on only one epitope for antibody binding, and/or that some individuals do not produce antibodies against that specific epitope. When maximum sensitivity is needed, sera should be prescreened using the PRNT prior to testing with the blocking ELISA.

Both the blocking ELISA and PRNT are highly specific for a given alphavirus or species. However, the blocking ELISA is superior in distinguishing infections with different VEEV subtypes and varieties that are known to have dramatically different abilities to amplify and spread via equine viremia and mosquito transmission. Our data indicated that the MAbs described by Roehrig and Bolin,[25] which distinguish epizootic from enzootic VEEV varieties, are useful in serologic assays in detecting variety-specific seroconversion in humans and animals. Establishing these assays using recombinant Sindbis/VEE viruses[35] and VEEV pseudotypes,[36] which are non-infectious or highly attenuated in animal models, and not regulated as select agents, would lower the risk of laboratory infections associated with VEEV use and simplify diagnostic procedures. These assays are under development in our laboratory.

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